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Molecular Epidemiology of an Outbreak of HCV in a Hemodialysis Unit: Direct Sequencing of HCV-HVR1 as an Appropriate Tool for Phylogenetic Analysis

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Infection with hepatitis C virus (HCV) is still a serious problem in hemodialysis patients, despite screening of blood products for anti-HCV antibodies. The prevalence of HCV in HD patients is between 15% and 30% in Germany. We report the molecular epidemiology of an HCV outbreak in a hemodialysis unit in 1997 is determined. HCV hypervariable region 1 (HVR1) was amplified from serum samples of 19 patients by polymerase chain reaction (PCR) and sequenced directly. In addition, HCV isolates from 3 of these 19 patients were cloned and sequenced. 14 newly infected patients and two patients, who had been infected for several years had very closely related HCV isolates. Unrelated HCV isolates as well as sequences obtained from an HCV outbreak in a plasmapheresis center were found in different, distantly related branches. These findings provide strong evidence for nosocomial transmission of the virus, despite following strict general hygiene precautions. The production of anti-HCV antibody was delayed significantly or seroconversion did not occur at all during the period of observation in 8 out of 14 newly infected HCV RNA positive patients. Close-meshed reverse transcription-polymerase chain reaction (RT-PCR) analyses on apparently non infected patients within hemodialysis units and upon admission of new patients is strongly recommended for the early detection and prevention of outbreaks of HCV. ***J. Med. Virol. 60:152–158, 2000.*** © 2000 Wiley-Liss, Inc.

KEY WORDS: anti-HCV; RT-PCR; nosocomial transmission; phylogenetic tree

INTRODUCTION

Despite screening of blood products for anti-HCV, there is still a high risk of infection with hepatitis C virus (HCV) for hemodialysis patients. The prevalence of infection increases with the duration of hemodialysis treatment, in part independently from transfusion events [Hardy et al., 1992; Muller et al., 1992; Kapoor et al., 1993; Knudsen et al., 1993; Niu et al., 1993].

There is much evidence for nosocomial transmission of HCV in hemodialysis units using molecular characterization of viral strains [Forns et al., 1997; Olmer et al., 1997; de Lamballerie et al., 1996; Stuyver et al., 1996; Zeuzem et al., 1996; Sampietro et al., 1995; Allander et al., 1994]. Following these studies, genotyping and sequencing of the hypervariable region 1 (HVR1) was used to monitor an HCV outbreak in a hemodialysis unit, which came to light following several seroconversions or positive polymerase chain reaction (PCR) results at the end of 1996.

MATERIALS AND METHODS

Patients

Thirty-three out-patients (21 males and 12 females, mean age 59 ± 14 years) were dialyzed in the same hemodialysis unit (patients HD01 to HD33). The majority of the patients were suffering from diabetes nephropathy or nephrosclerosis (27% and 21%, respectively). All serum samples were tested for markers of infection with hepatitis C virus and serum transaminases (AST (aspartat-amino-transferase), ALT (alanin-amino-transferase)) from the beginning of 1997 onwards. Retrospectively, data preceding PCR and antibody tests were available from another laboratory.

Serological Assays and Routine HCV RNA Testing

In 1997, anti-HCV antibodies were tested by fourth generation ELISA (enzyme linked immunosorbent assay) (MONOLISA® anti-HCV-PLUS, Sanofi Diagnostics Pasteur, France; including recombinant proteins from regions NS3 and NS4 as well as from the core

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region). Serum HCV RNA was tested by reverse transcription-polymerase chain reaction (RT-PCR) nested. RNA was isolated from 200 μl serum using the High Pure Viral RNA Kit (Boehringer, Mannheim, Germany). cDNA was synthesized from one fifth of the RNA with 15 U Superscript II-RT (GIBCO/BRL, Eggenstein, Germany) in the presence of RT primer and 20 U of ribonuclease inhibitor (RNA-guard, Pharmacia, Freiburg, Germany). Primers of the 5′ NCR were used for nested PCR amplification as described previously [Thomssen et al., 1992]. All experiments included appropriate positive and negative controls. HCV genotype was determined by a commercially available test (Inno LiPA, Innogenetics, Zwijndrecht, Belgium).

Nucleic Acid Sequence Analysis

For RT-PCR of the HCV hypervariable region 1, reverse transcription (RT) was carried out with 50 ng of the HCV genotype 1-specific primer HVR2. One fourth of the cDNA was used for nested PCR amplification. PCR was carried outfollowing standard conditions. All primers, expected fragment lengths and annealing temperatures are summarized in Table I. For direct sequencing of PCR products, excess nucleotides and primers were removed using QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Nucleotide sequences were determined for both strands with the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit on an ABI 373A DNA sequencer (Applied Biosystems, Weiterstadt, Germany) using the internal PCR primers.

Sequence analysis was undertaken using the programme package GCG (Wisconsin Sequence Analysis Package, Version 9.0, WI. Additionally, PCR products of patients HD02, HD03 and HD04 were cloned into TA cloning vector (Invitrogen, Groningen, The Netherlands) following the manufacturer's recommendations. DNA sequence analysis was carried out in each case for at least 8 clones.

RESULTS

HCV Antibody and PCR Tests

Until January 1997, 7 out of 28 patients had antibodies against HCV, and 6 were HCV RNA positive as well . Three patients were infected with HCV genotype 1a and two with genotype 1b. For the 2 other patients, (complete) genotyping was not possible, in one case because of negative PCR results, in the other case PCR products were only typable as genotype 1. Between January and September 1997, another 14 patients seroconverted to anti-HCV and/or had HCV RNA. In 8 of these 14 patients, HCV RNA for at least some time was the only marker of HCV infection. In 2 of the patients, patients HD01 and HD07, a positive RT-PCR preceded seroconversion by one and three months, respectively. In 7 patients only HCV RNA was detectable until September 1997 (Fig. 1). All HCV isolates of the new patients were genotype 1a. None of these patients has received blood products or underwent invasive procedures including endoscopy during the last two years. None of the medical staff of the hemodialysis unit was HCV antibody- or RNA-positive at any time.

Nucleotide Sequence Analysis

Sequence analysis was carried out with few exceptions with HCV isolates from two serum samples (per patient) taken at different time points. As a control, PCR amplification and sequencing was undertaken with HCV isolates generated from 3 patients from routine diagnostic, who were also infected with HCV genotype 1a. In addition, as a positive control for closely related isolates, HCV sequences from 14 patients (PP01 to PP14, genotype 1a) involved in an HCV outbreak in a plasmapheresis center in 1977 were included in phylogenetic analysis.

Phylogenetic analysis was undertaken with DISTANCES and GROWTREE from the GCG programme package using the Kimura-2-parameter distance matrix and Neighbor Joining. HCV sequences derived from all newly infected patients from the hemodialysis unit formed a distinct cluster in phylogenetic analysis, including HCV sequences from patients HD03 and HD19 (Fig. 2). Distances, calculated as unweighted substitutions per 100 nucleotides, were 5.8 ± 2.2 between sequences inside this cluster. Sequences derived from the HCV outbreak in the plasmapheresis center formed another cluster with comparable distances (4,8 ± 1,9) between particular isolates. Distances between different isolates of a single patient, taken at different time points, were 3,3 ± 2,0. Thus, there is no significant difference between distances between isolates from a single patient and HCV isolates

TABLE I. Primers Used for Reverse Transcription (RT), PCR and direct Sequencing (2nd PCR Primers)

Primer	Used for	3′ → 5′ position[a]	Fragment length	Sequence	Hybridization temperature
HVR1	1st PCR	1003–1022		GCTCAGCTGCTCCGGATCCC	
HVR2	1st PCR, RT	1395–1379	392 bp	CGGTAAGGCGTCGGCAGCTGGC	58°C
HVR3	2nd PCR	1077–1098		AGCGTATTTCTCCATGGTGGGG	
HVR4	2nd PCR	1278–1260	201 bp	GGCCGTGCTATTGATGTGCC	58°C
HVRI	1st PCR	1089–1108		TCCATGGTGGGGAACTGGGC	
HVRII	1st PCR, RT	1276–1254	187 bp	GTSCTRTTGATGTGCCARCTGCC	58°C
HVRIII	2nd PCR	1118–1138		GGTAGTGCTGCTGCTATTTGC	
HVRIV	2nd PCR	1241–1221	123 bp	CAGCTGGATGTTCTGCTTGGC	58°C

[a]Nucleotide positions are according to sequence XXX. Expected fragment lengths and hybridization temperatures used in PCR are given.

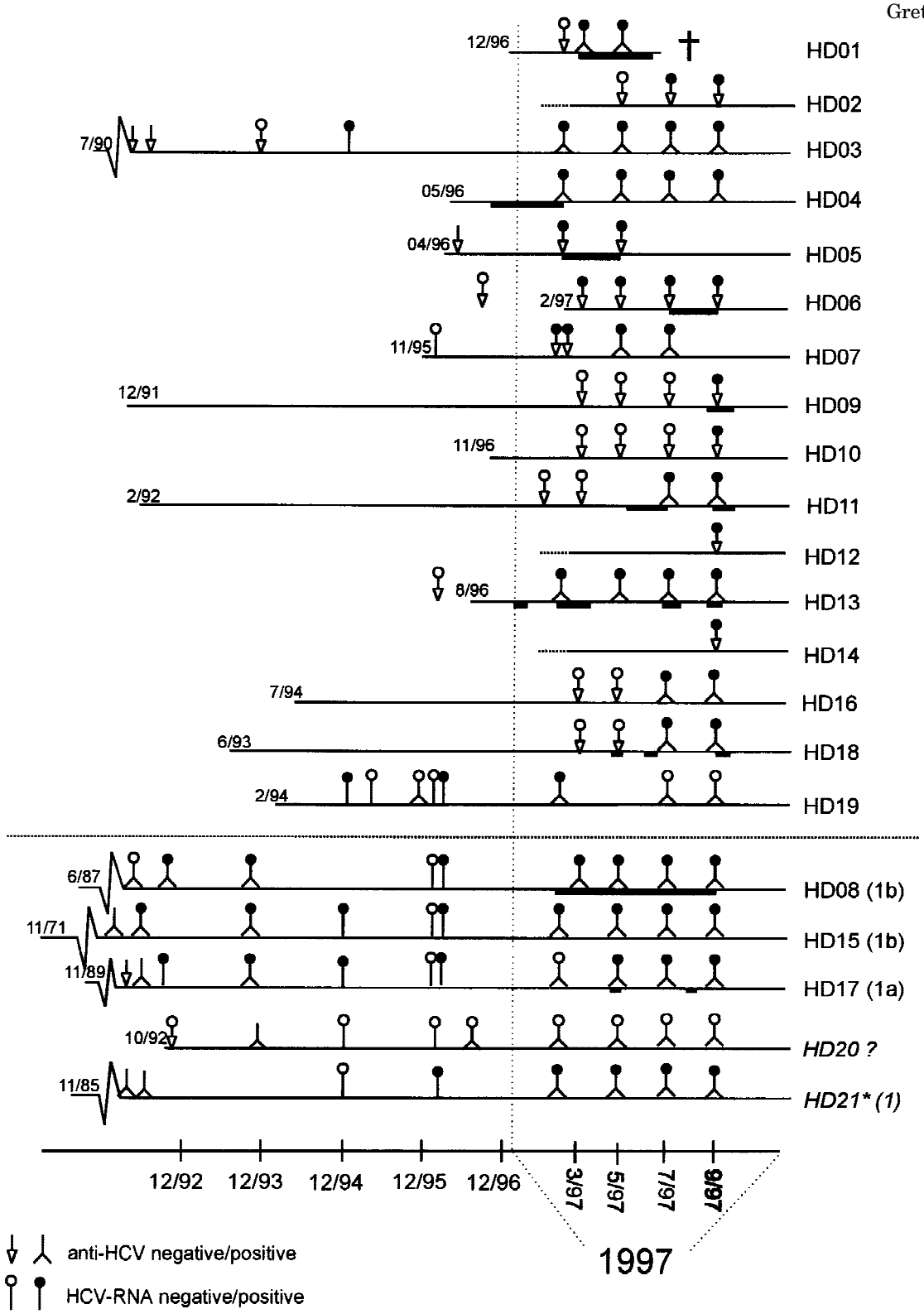

Fig. 1. Survey of all HCV positive patients of the hemodialysis unit. The horizontal lines give the time the patients have been dialyzed at the HD unit, the date at the left of each line is the month of the first dialysis. Informations about anti-HCV status and/or results of HCV RT-PCRs are depicted. Open triangles stand for anti-HCV positive samples, closed reversed triangles mean samples tested negative for anti-HCV, no triangle at all means anti-HCV not tested or data not available. HCV RNA positive samples are represented by filled circles, empty circles stand for HCV RNA negative samples, no circle means RT-PCR not done with this sample ore data not available. Elevated transaminase levels are given as thick lines. HCV isolates of patients above the horizontal dotted line were all genotype 1a and closely related by phylogenetic analysis. Isolates below that line presumedly did not belong to the actual outbreak. Genotypes are given in brackets. For patient HD20, genotyping could not be carried out because of negative RT-PCR results. For isolate HD21 subtyping was not possible, and, due to unknown reason, amplification of the HVR1 was not successful, either.

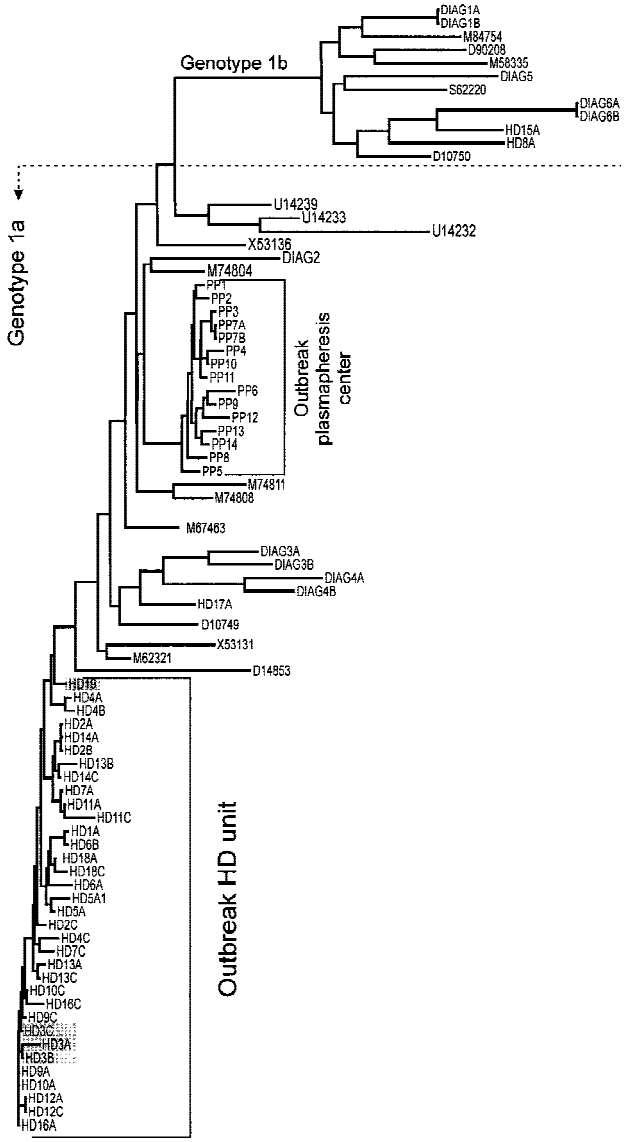

Fig. 2. Phylogram of HCV HVR1 sequences, created with DISTANCES and GROWTREE of the GCG program package using the Kimura-2-parameter matrix and Neighbor joining. HD01 to HD19: HCV isolates from patients of the HD unit. PP01 to PP14: HCV isolates from patients involved in an outbreak of HCV in a plasmapheresis center in 1977, all genotype 1a DIAG1 to DIAG6: HCV isolates from diagnostic samples not related to the outbreaks. DIAG2-4: genotype 1a; DIAG1, 5 and 6: genotype 1b. Sequences obtained from the EMBL nucleotide database: genotype 1a: d00831, d10749, d14853, m62321, m67463, m74811, m74804, m74808, u14232, u14239, u14233, x53131, x53136; genotype 1b: d10750, d90208, m58335, m84754, s62220. The clusters of the two outbreaks are marked. The HCV isolates of the two HD patients belonging to the HD outbreak, who have been already infected in 1994, are boxed.

from different patients within one outbreak (Fig. 3). This is illustrated by the fact that isolates from the same patient might be found in different branches inside the cluster formed by the outbreak sequences (Fig. 1). Isolate HD17, although genotype 1a, was found in an unrelated branch (distances to the isolates belonging to the outbreak: 18.6 ± 1.6). Distances between unrelated isolates of the same subtype were 22,7 ± 5,7 substitutions per 100 nucleotides (with a mean distance of 13 between the nearest related sequence m62321 and the isolates of the cluster). Genotype 1b-isolates from the hemodialysis unit (HD08 and HD15) were not related and differed in 24.2 positions per 100 nucleotides.

DISCUSSION

In this study, with sequence analysis of the HCV HVR1, strong evidence was obtained for nosocomial transmission of HCV in a hemodialysis unit, leading to an outbreak with 14 patients seroconverting to anti-HCV or with positive HCV-RNA during a nine month period. While all but two of the patients infected with HCV previously harboured unrelated viral strains, HCV isolates of all newly infected patients and patients HD03 and HD19, who had been infected in 1994, were related very closely by phylogenetic analysis. Therefore, direct sequence analysis of the HCV HVR1 was an appropriate tool for the analysis of recent transmission events, as described by others [Allander et al., 1994; Suzuki et al., 1994; Allander et al., 1995; de Lamballerie et al., 1996; Esteban et al., 1996].

Alternatively, some researchers analyzed a part of the NS-5b region alternatively [Healey et al., 1996], which, because of its lower variability can be used for the analysis of transmission events dating back for a longer time, ranging from several years to decades [Power et al., 1995; Munro et al., 1996]. For comparative reasons, a 220 bp-fragment of the NS-5b region [Simmonds et al., 1993] was also examined. Differences in distances between related and non related isolates were not as clear as with analysis of the HVR1; mean distances between unrelated isolates were about 4 substitutions per 100 nucleotides (ranging from 1 to 7), and 0.6 changes (ranging from 0 to 2.7) were found between isolates belonging to the outbreak, respectively. Because of the relatively large cohort studied above, by phylogenetic analysis all sequences belonging to the outbreak also formed a distinct cluster as well (phylogram not shown). There would have been some difficulties in considering only one pair of sequences. Taking these data into account, sequencing of the HCV HVR1 seems to be a more convenient tool for the determination of phylogenetic relationship when infection occurred recently, even when there are only few isolates from the same geographic region for comparison. These findings are in accordance with Smith and Simmonds [1997], who recommend analysis of the short hypervariable region for recent transmission events, while longer sequences of genomic regions like NS-5b should be the regions of choice for the analysis of transmission events, that occurred several years previously.

In this study, direct sequencing was carried out in order to reflect the relationship of HCV isolates. As outlined by Odeberg et al. [1995], this method allows for rapid determination of the consensus sequence of a heterogeneous virus population. In order to analyze

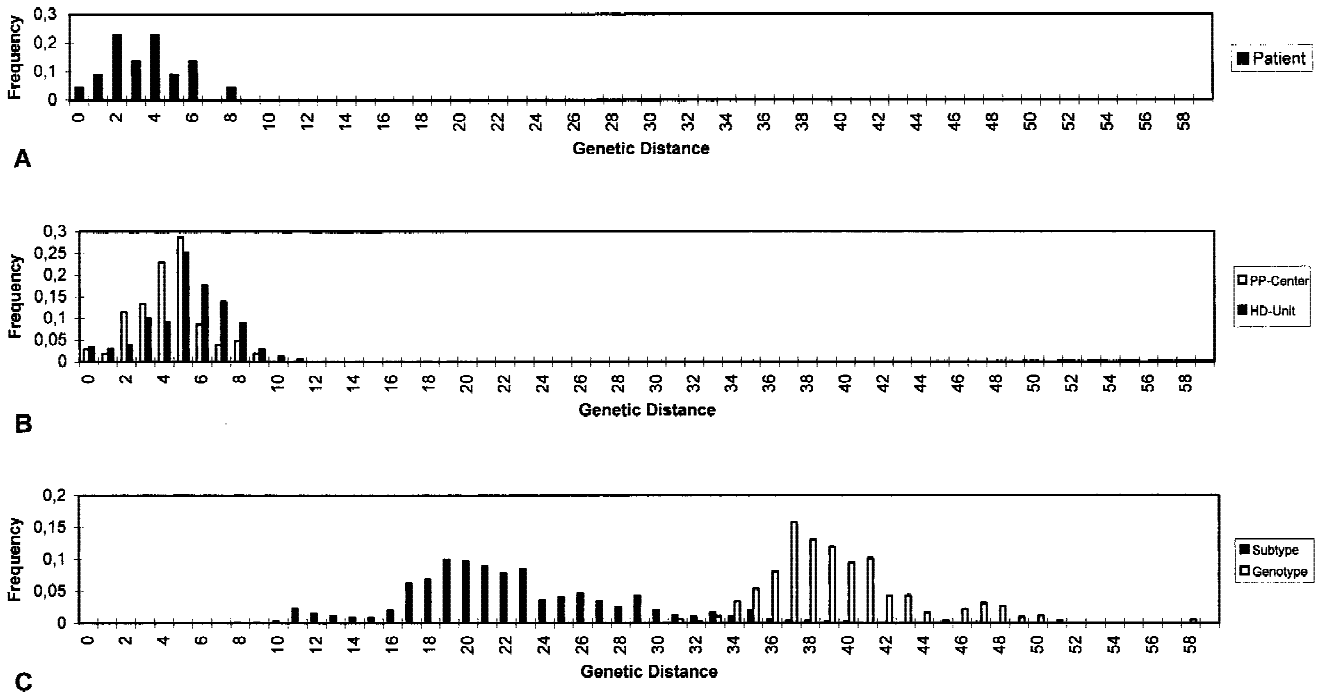

Fig. 3. Frequency distribution of genetic distances corresponding to different groupings: between **A:** different HCV isolates from single patients, taken at different timepoints within a period of 6 months (two or three isolates of patients HD 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 16, 18, respectively). **B:** different patients infected by strains within one cluster (strains from two outbreaks: hemodialysis unit, plasmapheresis center). **C:** unrelated HCV isolates from the same subtype (1a) and between unrelated isolates from the same genotype, but different subtypes (1a/b).

particular viral strains, at least 6 clones of the RT-PCR products were examined in each of two serum samples of isolates HD02, HD03 and HD04, respectively. The particular sequences reflected the quasispecies nature of HCV with many different viral strains, forming together the consensus sequence found by direct sequencing, including ambiguous positions (Fig. 4).

The period during which the infection occurred is estimated from etiologic data and spans from November 1996 at the latest to at least February 1997. Patient HD04 as the first patient infected had raised transaminase levels at the end of November 1996, and patient HD06, who became infected by the outbreak HCV strain as well, was first dialyzed in the HD unit in February 1997 (Fig. 1). The course of transmission remains unclear, but retrospective analysis provides the reason for the assumption that the outbreak has started from patients HD03 and/or HD19 who had been positive for HCV-RNA since at least December 1994. The transmission event between these two patients must have had taken place in 1994. Because of the closer phylogenetic relationship to the HCV isolates of the newly infected patients, the actual outbreak started more likely by patient HD03.

The causal event(s) that lead to the actual outbreak, could not be traced. Patients and medical staff were interviewed, but there was no obvious breakage of general hygiene precautions. Dialysis machines were autoclaved (Miro-Clav), disinfected with Maranon every day (Miro 1) or daily with 2,5 % acetic acid peroxide and once a week with Maranon (AK 100), respectively. Dialyzers were not reused and all nurses followed infection control measures, e. g., changing gloves before attending another patient. Hygiene precautions were checked by the public health office and considered sufficient. Nevertheless, there are several reports (reviewed by Sampietro et al., 1996) suggesting that ineffective (or occasional breaks in) infection control measures was the cause of nosocomial infections with HCV. However, the particular source of infections could not be clearly identified in any study. As a consequence, the importance of strict general hygiene precautions should be stressed again.

Focusing on the particular outbreak, it was striking, that in 8 out of 14 patients with HCV RNA, seroconversion was delayed or did not occur during the period of observation. Other researchers also report delayed, diminished or absent antibody-reactivity against HCV antigens in hemodialysis patients [Caramelo et al., 1996; Gerken et al., 1996; Lee et al., 1996; Pujol et al., 1996; Abdelnour et al., 1997; Fabrizi et al., 1998]. These findings imply that HCV seroprevalence in hemodialysis units underestimates the actual prevalence of infection. Because of the high risk of HCV transmission in hemodialysis units even through unrecognized cases, regular close-meshed RT-PCR analyses are recommended for apparently non-infected patients within a hemodialysis unit as well as on admission of new patients.

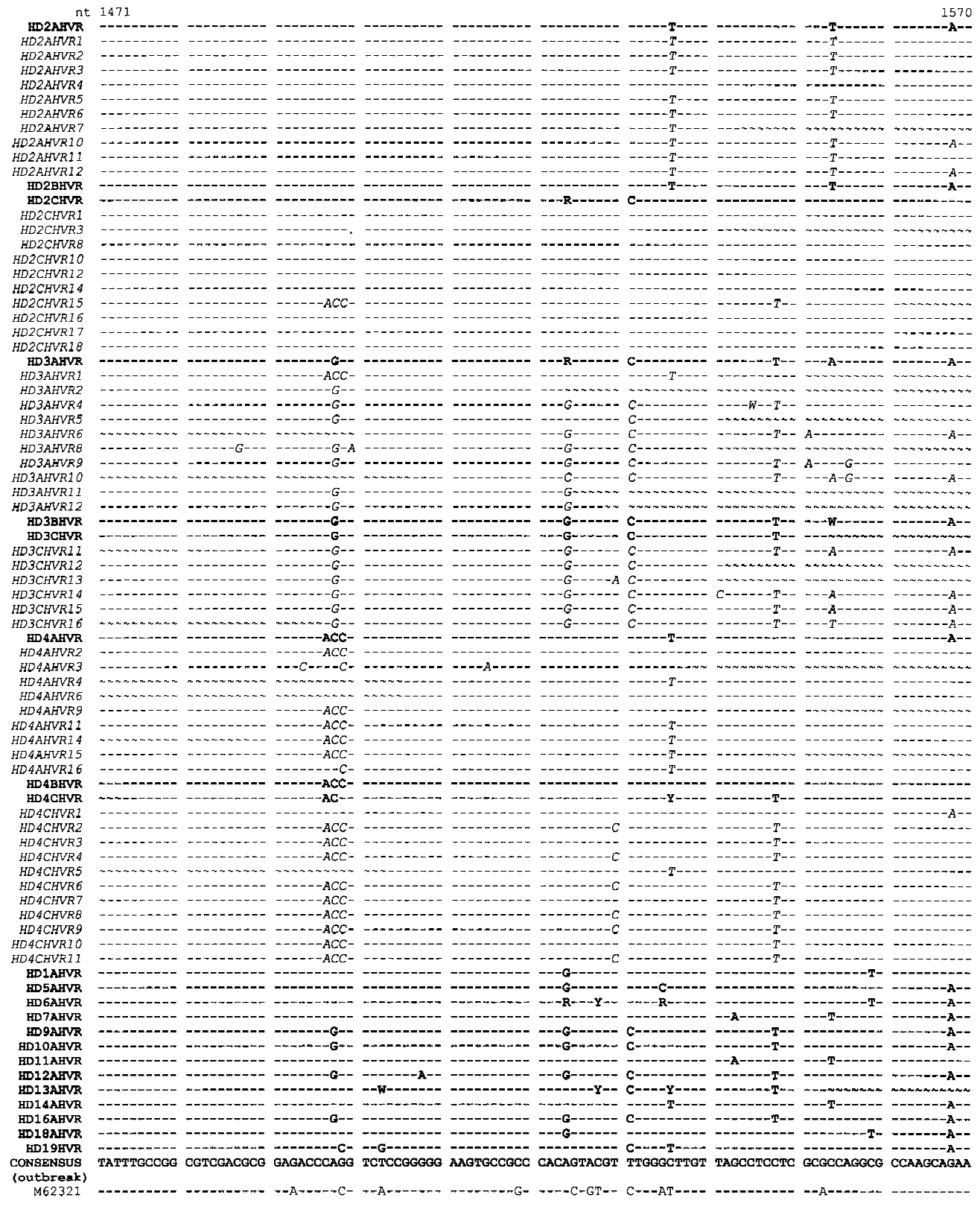

Fig. 4. Alignment of a 100 nucleotide fragment around HCV HVR1 of HCV isolates belonging to the outbreak in the HD unit. Isolates sequenced directly are printed in bold, cloned isolates are given in italics. In the cases where no clones were analyzed, HCV isolates of the patients are represented by the one amplified from the first HCV positive sample. For comparison, sequence m62321 as the phylogenetically closest related sequence is given as well.

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